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Thurnheer, Thomas ; Belibasakis, Georgios N ; Bostanci, Nagihan

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Colonization of gingival epithelia by subgingival biofilms *in vitro*: role of "red complex" bacteria

Thomas Thurnheer[a *], Georgios N. Belibasakis[a], Nagihan Bostanci[b]

[a] Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Switzerland

[b] Oral Translational Research, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Switzerland

Running title: Colonization of gingival epithelia by subgingival biofilms

[*] **Corresponding Author:**
Dr. Thomas Thurnheer, PhD
Oral Microbiology and Immunology
Institute of Oral Biology
Center of Dental Medicine
University of Zürich
Plattenstrasse 11
8032 Zürich
Switzerland
E-mail: thomas.thurnheer@zzm.uzh.ch
Tel: +41-44-6343376
Fax: +41-44-6343310

Abstract

Objectives: Biofilm formation on tooth surface results in colonization and invasion of the juxtaposed gingival tissue, eliciting strong inflammatory responses that lead to periodontal disease. This *in vitro* study investigated the colonization of human gingival multi-layered epithelium by multi-species subgingival biofilms, and evaluated the relative effects of the "red complex" species (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*).

Methods: The grown biofilm consisted of *Fusobacterium nucleatum*, *Campylobacter rectus*, *Veillonella dispar*, *P. gingivalis*, *Prevotella intermedia*, *T. forsythia*, *T. denticola*, *Actinomyces oris*, *Streptococcus anginosus* and *Streptococcus oralis*, or its variant lacking the "red complex". After 48 in co-culture with the gingival epithelia, the bacterial species in the biofilm were quantified, whereas their localization on the cell surface was investigated by combining confocal-laser scanning microscopy (CLSM) and fluorescence *in situ* hybridization (FISH), as well as by scanning electron microscopy (SEM).

Results: Exclusion of the "red complex" quantitatively affected *S. oralis*, but not other species. The "red-complex" species were all able to colonize the gingival epithelial cells. A co-localization trend was observed between *P. gingivalis* and *T. denticola*, as determined by FISH. However, in the absence of all three "red complex" bacteria from the biofilm, an immense colonization of streptococci (potentially *S. oralis*) was observed on the gingival epithelia, as confirmed by both CLSM and SEM.

Conclusions: While the "red complex" species synergize in colonizing gingival epithelia, their absence from the biofilm enhances streptococcal colonization. This antagonism with streptococci reveals that the "red complex" may regulate biofilm virulence, with potential implications in periodontal pathogenesis.

1. Introduction

Periodontitis is among the most common infectious inflammatory diseases in humans. It is characterized by the destruction of the tooth supporting (periodontal) tissues, as a result of an excessive chronic local inflammatory response of the tissues, eventually leading to tooth loss if left untreated. These events are caused by oral bacteria colonizing the tooth surfaces in the form of polymicrobial biofilm communities. Failure to eliminate the biofilms from the tooth surface by means of proper oral hygiene will eventually result in their growth below the gingival margin, whereby they become "subgingival" and interact with the juxtaposing periodontal pocket epithelium [1]. The developing tissue inflammatory response aims to prevent bacterial colonization and establishment, but if it becomes excessive, the inflammation will lead to tissue destruction rather than being protective [2, 3]. Characteristic bacteria of the subgingival biofilm are the Gram-negative anaerobes *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, which are all associated with periodontal disease rather than health. These are also clustered as the "red complex" species [4].

The gingival epithelium of the periodontal pocket is the first tissue to interact with the bacterial components of the subgingival biofilm. This interaction involves bacterial colonization and invasion of the tissue and the initiation of the inflammatory responses that eventually lead to the pathogenesis of periodontitis. Oral or gingival epithelial cells can be colonized and invaded by putative periodontal pathogens, such as *P. gingivalis*, *T. forsythia*, *T. denticola* and *Fusobacterium nucleatum* in single and mixed infections [5].

Studying *in vivo* the interaction between subgingival biofilms and periodontal tissues, such as the gingival epithelium, is cumbersome and poses several ethical and technical limitations. The development of the 10-species *in vitro* biofilm model (also known as the "Zürich" subgingival biofilm) permitted the experimental co-culture with host cells, in a manner that mimics the host-biofilm interface of the periodontal pocket environment [6]. Thus, studying aspects of this interaction became possible *in vitro*. This biofilm model has been shown to

induce pro-inflammatory cytokine production in gingival epithelial cells [6, 7] and osteolytic mediators, such as receptor activator of NF-kB ligand (RANKL) and prostaglandin E_2, in gingival fibroblasts [8, 9]. It has also been shown to down-play pathogen-sensing inflammasome machinery in gingival fibroblasts [10], an event largely attributed to *P. gingivalis* [11]. Although focus has been on how this subgingival biofilm regulates host inflammatory responses, the cell-colonizing behavior of the involved species has not yet been investigated. Moreover, the relative contribution of the three "red complex" species on the quantitative composition or the colonizing properties of the remaining biofilm species, when in co-culture with host cells, has not been evaluated.

Hence, this *in vitro* study employed a co-culture of human gingival multilayered epithelia and the 10-species subgingival biofilm, or its 7-species variant lacking *P. gingivalis*, *T. forsythia* and *T. denticola*. The aim of the study was to investigate the relative effects of these three "red complex" species on the quantitative composition and cell-colonizing capacities of the remaining seven species. It is hypothesized that the composition and colonization capacity of the biofilm may selectively be affected in the absence of the "red complex", implying a role in regulating the virulence of the biofilm.

2. Materials and methods

2.1. Biofilm generation

Subgingival biofilms used in this study were produced using a modified protocol [6, 12] as described elsewhere [13]. In brief, multispecies biofilms were grown in medium, consisting of 60% of processed whole unstimulated pooled saliva, 30 % modified fluid universal medium (mFUM) [14] and 10 % heat inactivated human serum. Incubation was for 64 h under anaerobic conditions at 37 °C. The biofilm consortium was composed of *Streptococcus oralis* SK248 (OMZ 607), *Streptococcus anginosus* ATCC 9895 (OMZ 871), *Actinomyces oris* (OMZ 745; formerly

Actinomyces naeslundii), *Fusobacterium nucleatum* subsp. *nucleatum* OMZ 598, *Veillonella dispar* ATCC 17748^T (OMZ 493), *Campylobacter rectus* OMZ 698, *Prevotella intermedia* ATCC 25611^T (OMZ 278), *Porphyromonas gingivalis* ATCC 33277^T (OMZ 925), *Tannerella forsythia* OMZ 1047, and *Treponema denticola* ATCC 35405^T (OMZ 661). Alternatively, to evaluate the relative involvement of the "red complex" species, a biofilm variant whereby *T. denticola*, *P. gingivalis* and *T. forsythia* had been omitted, was also grown. All strains were maintained on Columbia Blood Agar (CBA) plates, with the exception of *T. forsythia* and *T. denticola* that were maintained in liquid growth media as described previously [12]. To ensure a high viability in the pre-culture phase, *C. rectus* was incubated for 64 h in liquid medium under microaerophilic conditions prior to experiments. Two cycles of pre-cultures were performed for all strains prior to the inoculation of the biofilms: Bacteria were transferred into the corresponding liquid growth medium described earlier [12], and incubated for 24 h (cycle 1). Following this, the pre-cultures were diluted 1:10 fresh medium and incubated for another 8 h (cycle 2). Following the pre-culturing, all cultures were set to a defined optical density (OD_{550} = 1.0 ± 0.05) and mixed in equal volumes. Prior to the inoculation, sintered hydroxyapatite discs were placed in a 24-well plate and incubated for 4 h at room temperature in 800 µl of pooled saliva on a rotary shaker (90 RPM) in order to form a pellicle. The discs were then placed into 24-well plates containing 1.6 ml of growth medium per disc, followed by the inoculation using 200 µl of the bacterial mixture for each disc. After 16 h of incubation the growth medium was renewed, along with adding 50 µl of *T. denticola* liquid culture (OD_{550} = 1.0). In the following, the growth medium was renewed every 24 h until the full incubation time of 64 h was reached.

2.2. Multilayered gingival epithelial culture interaction with the *in vitro* biofilm

The stratified gingival organotypic epithelial cell cultures used in this study were received in 24-well plate format (0.5 cm² surface), and were commercially available (EpiGing, MatTek, Ashland,

MA, USA). This in vitro tissue expresses cytokeratin K13 and K14, as well as human beta defensin (HBD) HBD-1 and only weakly HBD-3. It is composed of normal human gingival epithelial cells cultured to form a highly differentiated multi-layered tissue with keratinized layers, morphologically resembling the gingival epithelium (Fig. 1). They were maintained in culture in defined keratinocyte serum-free medium (K-SFM), supplemented with 0.05 mM calcium chloride and 200 mM L-glutamine (Gibco/Invitrogen, Lucerne, Switzerland). The experiments with the biofilms were all performed under aerobic conditions. For the experimentations, the HA-discs carrying the 64.5 h grown biofilm were put in a plastic ring that had the same diameter as the HA-disc, but exceeded its height by 1 mm on the biofilm side. Thereafter, the discs with the ring were put upside-down on the multilayered epithelial cell culture, whereupon the plastic ring prevented the biofilm from having direct contact with the epithelial cells. The exposure times of the multilayered epithelial cell culture to the biofilms were 24 h and 48 h and were carried out under aerobic conditions. Then, biofilms were either harvested for culture or microscope analysis as previously described [13].

2.3. Staining procedures

After completion of the experiments, the 3D-gingival epithelium was stained using the PKH67 Green Fluorescent Cell Linker Kit for general cell membrane labeling, fluorescein isothiocyanate labeled phalloidin for F-actin labeling and DAPI for nuclear DNA staining (all from Sigma-Aldrich Chemie, Buchs, Switzerland), according to the manufacturer's instructions, in 24-well culture dishes. Biofilm cells were stained by multiplex FISH using species-specific probes following the protocols described before [12, 15]. Pre-hybridization (15 min, 46 °C) was performed in 500 μl hybridization buffer without probes added. Five hundred μl of hybridization buffer was used for each biofilm, supplemented with up to 3 probes at a concentration of 10 ng/μl for each. The incubation time for the hybridization was at least 3 h at 46 °C in the dark. After the incubation,

biofilms were transferred into washing buffer pre-heated to 48 °C and incubated for 20 min at this temperature. After hybridization, the samples were embedded upside-down on chamber slides in 100 μl of Mowiol [16]. Probe sequences and formamide concentrations used for the hybridizations, as well as the NaCl concentrations of the washing buffers are given in Table 1.

2.4. Confocal laser scanning microscopy (CLSM)

The use of CLSM on cell cultures was performed as described previously for eukaryotic cells [17]. In brief, stained cell culture samples were examined using a Leica SP-5 inverse confocal microscope (Leica Lasertechnik, Heidelberg, Germany), fitted with a UV laser (405 nm excitation), an Ar laser (458 nm / 476 nm / 488 nm / 496 nm / 514 nm excitation), and a He-Ne laser (561 nm / 594 nm / 633 nm excitation). Filters were set to 430-470 nm to detect DAPI, to 500–540 nm for FITC, to 570–630 nm for Cy3, and to 660–710 nm for Cy5. Confocal images were obtained using x 63 (numeric aperture 1.30) glycerol immersion objective. Z-series were generated by vertical optical sectioning with the slice thickness set at 1.02 μm. Image acquisition was performed in x 8 line average mode. Scans were recombined and processed using Imaris 7.3.0 software (Bitplane, Zürich, Switzerland), without any qualitative changes to the raw images.

2.5. Scanning electron microscopy (SEM)

For the preparation of the SEM images, the biofilm disc were removed from the gingival epithelial cultures, and washed twice with PBS. The culture samples were then fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 1 h. They were thereafter washed again in PBS and dehydrated in a graded series of acetone. Following critical point drying, they were mounted on stubs and finally coated with 3 nm of Carbon. Imaging was performed on a Vega TS 5136 XM (Tescan, Dortmund, Germany) scanning electron microscope.

2.6. Histological staining

For histologically viewing the multiple gingival epithelial layers, the cultures were fixed in 2.5% glutaraldehyde for 2 h, followed by washing and post-fixation in 1% OsO_4 for 1.5 h (all in 0.185M Sodium cacodylate buffer). The samples were then dehydrated in a graded series of ethanol. Propylene oxide was used as a transition agent before embedding in Epon 812 (Fluka, Buchs, Switzerland). The 4 µm sections were obtained using a diamond knife on a Reichert-Jung Ultracut E Ultramicrotome (Leica Microsystems, Heerbrugg, Switzerland), floated on a 1% toluidine blue with 1% borax solution for 3 min at 60°C. Thereafter, they were mounted on glass slides. Finally, they were viewed with a DM6000 B microscope (Leica Microsystems, Heerbrugg, Switzerland). Representative histological images are provided in Fig. 1 (A: control, B: after biofilm challenge).

2.7. Statistical analysis

To investigate differences in the numbers of the remaining bacterial species in the absence of the three “red complex” species, a Two-way analysis of variance (ANOVA) was performed, using Sidak’s multiple comparisons test. Differences were considered statistically significant at $p<0.05$.

3. Results

The bacterial composition of the biofilms, with or without the “red complex” species, was first investigated, after 24 h and 48 h of co-culture with the multilayered gingival epithelium. Quantitatively, there were no differences in biofilm composition in the remaining species after 24 h or 48 h exposure time. The exception was *S. oralis*, which showed significantly decreased numbers in the absence of the “red complex” at 24 h (Fig. 2A), followed by a significant increase at 48 h (Fig. 2B).

On the other hand, the biofilm affected the gingival epithelium, when in co-culture. The gingival epithelial surfaces were colonized by the bacteria of the biofilm over a period of 48 h (Fig. 3). In the presence of the red complex species (Fig. 3B) there was a sparse but consistent colonization of the outer epithelial layer. When the red complex was omitted, a more pronounced bacterial colonization of the surface was observed (Fig. 3C). In closer view, the predominating bacterial cells appeared to be coccoid (Fig. 3D).

These gingival epithelial tissues in co-culture with the biofilms were further analyzed by CLSM. As seen from Fig. 4A, the epithelial tissue was unaffected after 24 h and 48 h, in the absence of the biofilms. However, when exposed to biofilm, the epithelial cells were invaded by the individual species (Fig. 4C and 4D). In particular, after 48 h of exposure to the biofilm, the epithelial tissue structure as well as the cell nuclei exhibited signs of degradation (Fig. 4D and 5B). As demonstrated in Fig. 4 and Fig. 5, all three “red complex” species were independently able to colonize the gingival epithelial tissue, whereas *P. gingivalis* and *T. denticola* appeared to co-localize on the epithelium (Fig. 4A). Interestingly, in the absence of the “red complex” species, a mostly coccoid microbiota seemed to predominate on the epithelial layers after 48 h (Fig. 4D), which proved to be mainly streptococci (Fig. 6B). Nevertheless, this excessive streptococcal colonization was not observed when the “red complex” species were present in the composition of the biofilm (Fig. 5A and 6A). This finding is well in line with the observations by SEM (Fig. 3).

4. Discussion

Bacterial colonization and invasion of gingival tissue is a crucial property for bacterial survival and a key aspect in the pathogenesis of inflammatory periodontal diseases. The present study has used CLSM to investigate the colonizing interactions between subgingival biofilms and multi-layered gingival epithelial cultures *in vitro*. It is well established that all three red complex

species can colonize gingival epithelial cells, and this has been also demonstrated in the present experimental system, as constituents of a biofilm community.

Yet, little is known on the interaction of different oral bacterial species with regards to the regulation of their capacity to colonize structured multilayered gingival epithelia. It has been reported that *F. nucleatum* can invade organotypic epithelial tissue *in vitro*, and its invasive capacity is higher when cultured in biofilm, rather than in planktonic form [18]. Also, *F. nucleatum* facilitates the colonization of *P. gingivalis* [19, 20]. In its own right, *P. gingivalis* has a well-recognized capacity to invade epithelial cell and penetrate deeper into periodontal tissues, thus evading the innate immune responses [21]. It can also synergize or antagonize other periodontal pathogens. For instance, *T. forsythia* can attenuate the invasion of gingival epithelial cells by *P. gingivalis* [19], while *P. gingivalis* may conversely enhance the invading capacity of *T. forsythia* [22]. These two species are shown to co-localize in the intracellular compartment of the oral epithelial cells that they have invaded into [23]. Earlier studies did not detect invasion of *T. denticola* in multilayered organotypic epithelium, but found that, instead, its proteases are transferred intracellulalry in vacuoles within the cells [24]. Others show that it invades gingival epithelial cells, but rarely co-localized with either endosomes or lysosomes, potentially resisting endo-lysosomal degradation. In the present study it is shown that *P. gingivalis* and *T. denticola* appeared to co-localize on the gingival epithelial cell layers, a finding that corroborates recent data showing that these two species are spatially associated within this *in vitro* biofilm model [12, 25]. This statement is also supported by a study of Zhu et al. [26] who observed a strong synergy between *P. gingivalis* and *T. denticola* in multispecies biofilm formation. This finding may denote a synergistic interaction between these two taxa as part of the biofilm community, with regards to their cell-colonizing properties.

Nevertheless, the hallmark of the present study has been the investigation of the role of the three “red complex” species collectively, in the capacity of the remaining seven species of the biofilm to colonize the multilayered gingival epithelial cultures. It was demonstrated that

absence of the “red complex” from the biofilm composition caused an immense colonization of the gingival epithelial layers, by cocoid cells, predominantly streptococci. In the absence of nutritional or space competition imposed by the “red complex”, the fast growth rate of streptococci may allow them species to dominate over the remaining species of the biofilm. Two streptococcal species are included in the composition of the present 10-species biofilm model, namely *S. oralis* and *S. anginosus*. There is evidence that planktonic cultures of *S. oralis* can invade *in vitro* monolayers of human umbilical vein endothelial cells (HUVEC) [27], or human aortic endothelial cells (HAECs) [28, 29], whereas *S. anginosus* has a very restricted invasive capacity [29]. To date there is no evidence of the colonization capacity of these two streptococci on gingival epithelial cells, neither in planktonic state, nor as part of multispecies biofilms. Given the fact that *S. oralis* is shown to be invasive among these two streptococci, it is likely that the enhanced colonization of coccoid bacteria observed here, represents indeed *S. oralis*. This is further strengthened by the finding that, after 24 h of co-culture, the CFUs of *S. oralis* present in the biofilm are significantly lower when the “red complex” species are absent. The likely explanation for this numerical reduction is that *S. oralis* cells have departed from the biofilm, subsequently colonizing the juxtaposing layers of the gingival epithelial cells, after 48 h. A recent in vivo study in mice [30] supports these results; when specific-pathogen-free mice were inoculated with *P. gingivalis* changes in the composition of the microbiota and especially an increase in *Streptococcus* species were observed when compared to control animals.

In conclusion, the present study employs a complex multi-species biofilm-multilayered gingival epithelial cell interaction model, to demonstrate the colonization capacity of the individual bacteria. Among the red complex species, *T. denticola* and *P. gingivalis* appear to co-localize on the epithelial cells, denoting a synergistic interaction. Absence of the three “red complex” species from the biofilms enhanced the colonization of epithelial cells by coccoid bacteria, potentially *S. oralis*, denoting an antagonistic interaction. Hence, the presence of the “red complex” species may exert an inhibitory role on the colonization capacity of other bacterial

species in the biofilm. In this manner, they may be able to regulate the virulence properties of the whole biofilm community, but their precise mechanism of action awaits further clarification. This feature may well be crucial for the early steps of the pathogenesis of periodontal disease.

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Legend to figures:

Fig. 1 - Histological view of the multi-layer gingival epithelial cell cultures, following toludine blue staining. Control cultures (A) and cultures challenged with the biofilm (B) for 48 h. Notice the multiple epithelial layers, with the keratinized layers on top. The bacteria of the biofilm are also visualized in the biofilm-challenge group (B), having colonized the epithelial surface. Scale = 50 µm

Fig. 2 - Box plots showing cell numbers/biofilm with and without "red-complex" bacteria after 24 h (A) and 48 h (B) exposure to epithelial cells. Data represents mean ± SD from two independent experiments, each represented in triplicate cultures. Only *S. oralis* showed significantly decreased numbers in the absence of the "red complex" at 24 h (A, *$p<0.05$), followed by a significant increase at 48 h (B, *$p<0.05$).

Fig. 3 - Scanning electron microscopy images of the gingival epithelial cultures after 48 h, in the absence (A) or presence of the 10-species biofilm (B) or the 7-species biofilm where the "red complex" species have been excluded (C and D; magnification). Scales = 10 µm (A-C) and 2 µm (D).

Fig. 4 - CLSM images of gingival epithelial cells. Controls without exposure to biofilm after 24 h (A) and 48 h (B), respectively; after 48 h exposure to whole (10-species) biofilm (C) and biofilm without "red complex" species (D). Epithelial cells appear green due to membrane staining with PKH26 green fluorescent cell linker-kit, the nucleus is blue due to DAPI staining and bacteria appear light blue due to FISH staining using the universal bacterial probe EUB338. Due to additional FISH staining with L-Pgin1006-2 and TrepG1_679, *P. gingivalis* and *T. denticola*, respectively (C) are stained red. Scales = 20 µm.

Fig. 5 - CLSM images of gingival epithelial cells after 48-h exposure to the 10-species subgingival biofilm (A) and to the 7-species biofilm without red complex (B). Nuclei appear blue due to DAPI staining, cell membranes green due to staining with PKH26 Green Fluorescent Cell Linker-Kit. Bacteria were stained by FISH using probes EUB338 for total biofilm bacteria (light blue), and Tfor997 for *T. forsythia* (red). Scales = 20 µm (A) and 30 µm (B).

Fig. 6 - CLSM images of gingival epithelial cells after 48-h exposure to the 10-species subgingival biofilm (A) and to the 7-species biofilm without “red complex” (B). Nuclei appear blue due to DAPI staining, cells green due to F-actin staining with phalloidin. Bacteria were stained by FISH using probes EUB 338 for total biofilm bacteria (white), STR405 for total streptococci (red) and Fnuc133c for *F. nucleatum* (red). Scales = 10 µm.

Table 1: Oligonucleotide probe sequences, labels and formamide concentrations for FISH analyses

Organism	Name	Type	Labels	FA[1] (%)	WB[2] (mM)	Sequence (5' → 3')	Refs
F. nucleatum	Fnuc133c	DNA	Cy3, Cy5	40	46	GTTGTCCCTANCTGTGAGGC	6
P. gingivalis	L-Pgin1006-2	LNA[3]	Cy3, Cy5, FAM	30	112	GTTTTCACCATCMGTCATC	6
Streptococci	STR405	DNA	Cy3, Cy5	20	215	TAGCCGTCCCTTTCTGGT	31
T. denticola	TrepG1_679	DNA	Cy3, Cy5, FAM	40	46	GATTCCACCCCTACACTT	32
T. forsythia	Tfor997	DNA	Cy3, Cy5, FAM	40	46	TCACTCTCCGTCGTCTAC	33
biofilm bacteria	EUB338	DNA	Cy3, Cy5, FAM,	40	46	GCTGCCTCCCGTAGGAGT	34

[1]Formamide concentration in the hybridization buffer
[2] Concentration of NaCl used in the washing buffer
[3] Probes containing locked nucleic acid substitutes (LNA). The ribose ring of LNA is constrained by a methylene linkage between the 2' oxygen and the 4' carbon.

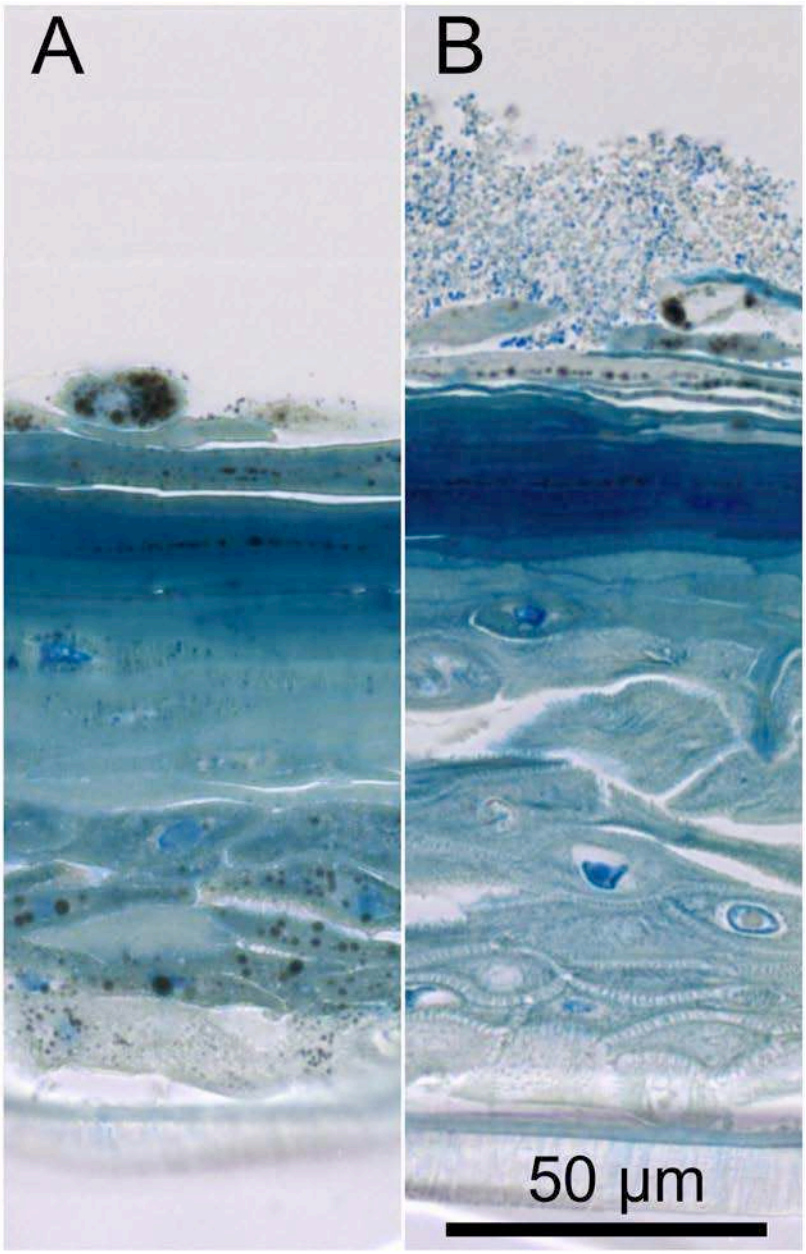

Fig. 1 - Histological view of the multi-layer gingival epithelial cell cultures, following toludine blue staining. Control cultures (A) and cultures challenged with the biofilm (B) for 48 h. Notice the multiple epithelial layers, with the keratinized layers on top. The bacteria of the biofilm are also visualized in the biofilm-challenge group (B), having colonized the epithelial surface. Scale = 50 µm.

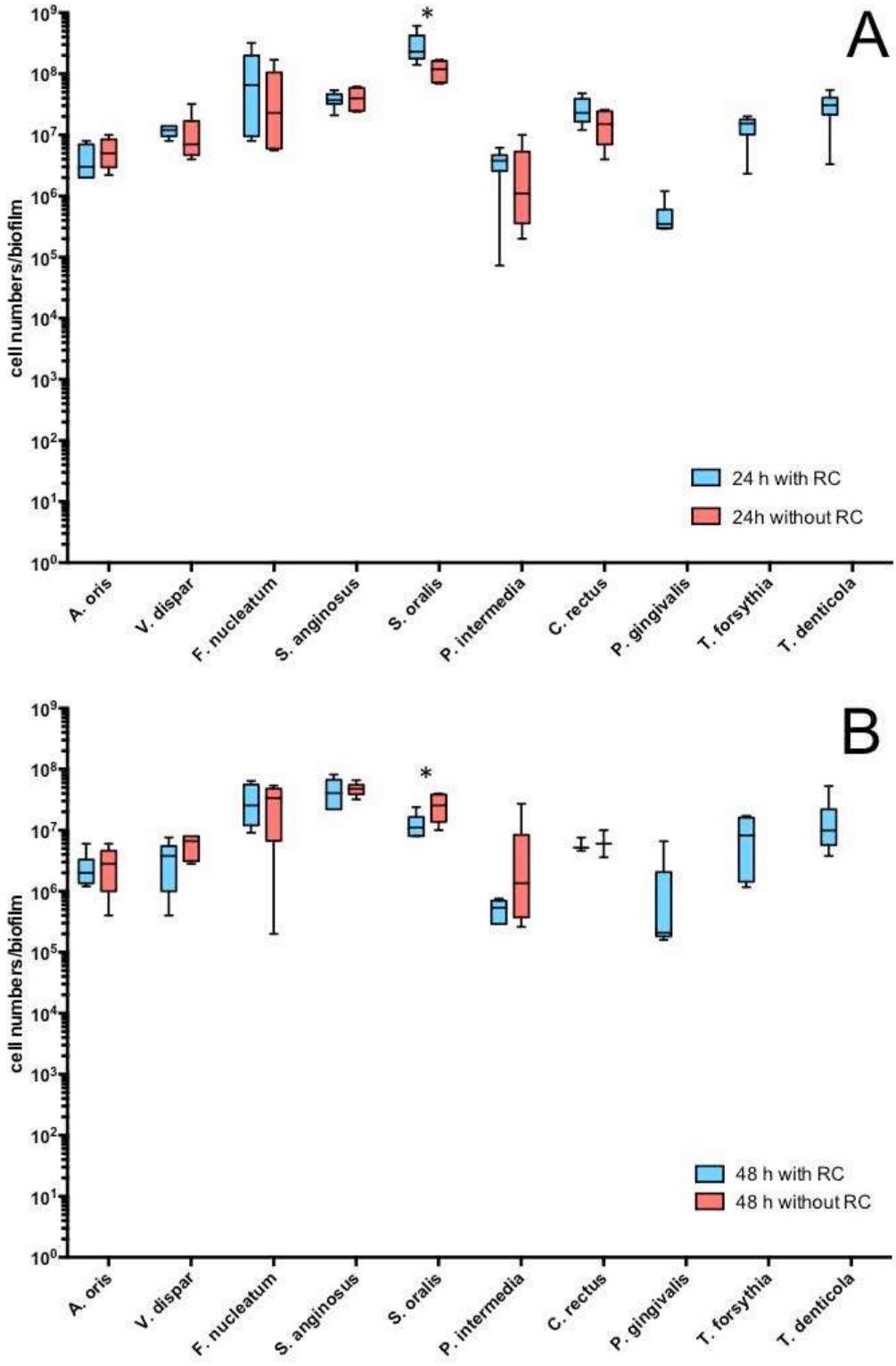

Fig. 2 - Box plots showing cell numbers/biofilm with and without “red-complex” bacteria after 24 h (A) and 48 h (B) exposure to epithelial cells. Data represents mean ± SD from two independent experiments, each represented in triplicate cultures. Only *S. oralis* showed significantly decreased numbers in the absence of the “red complex” at 24 h (A, *p<0.05), followed by a significant increase at 48 h (B, *p<0.05).

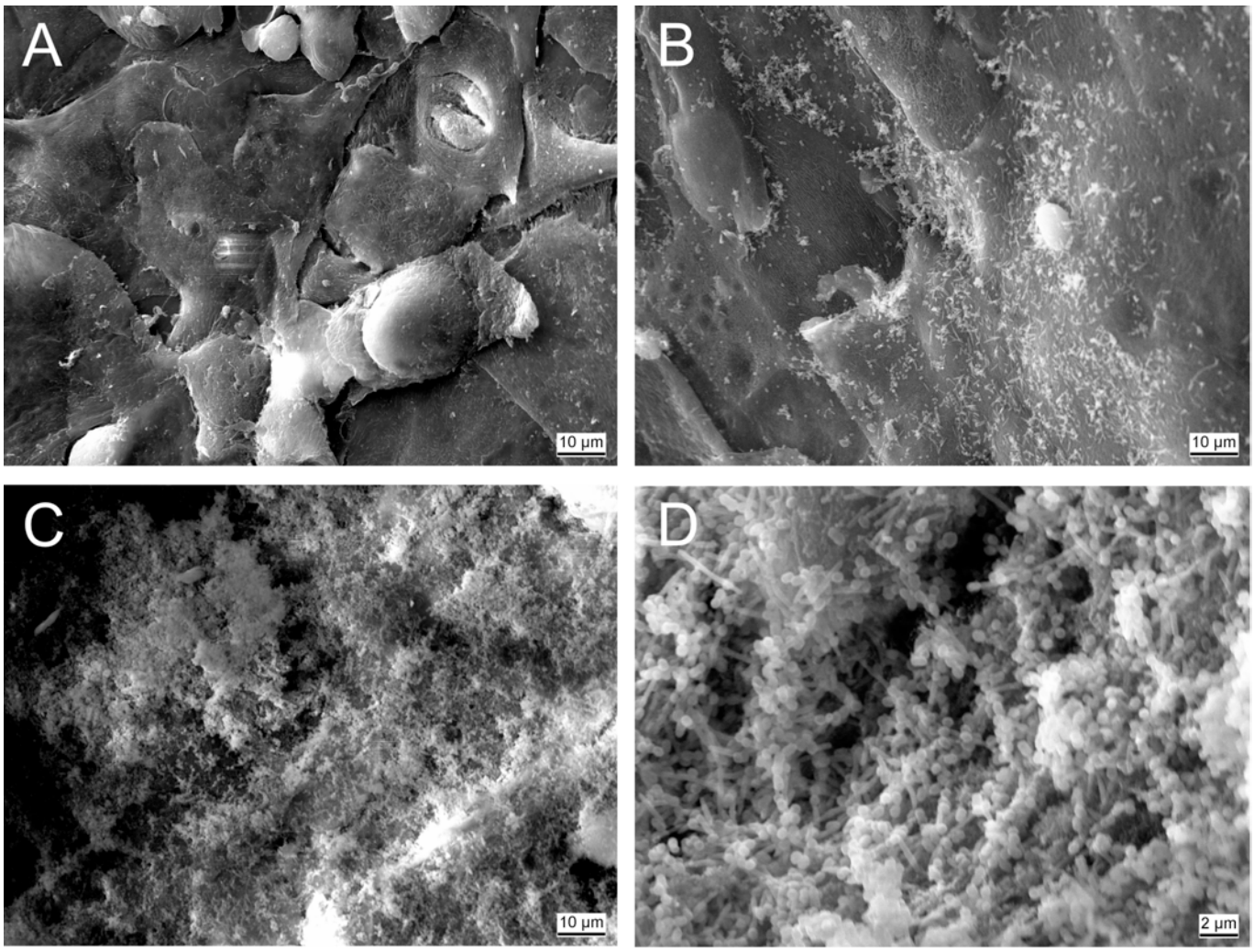

Fig. 3 - Scanning electron microscopy images of the gingival epithelial cultures after 48 h, in the absence (A) or presence of the 10-species biofilm (B) or the 7-species biofilm where the “red complex” species have been excluded (C and D; magnification). Scales = 10 µm (A-C) and 2 µm (D).

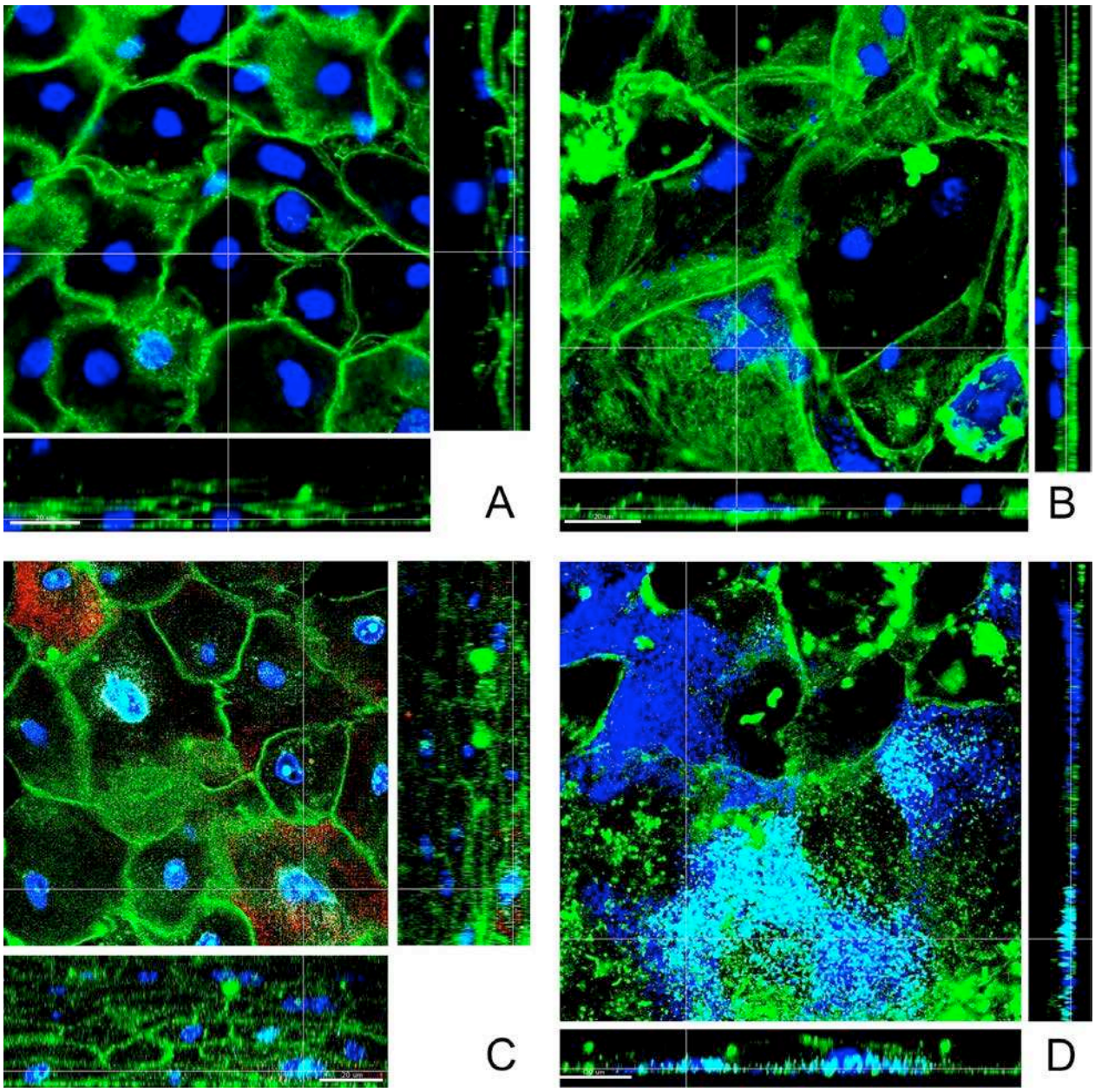

Fig. 4 - CLSM images of gingival epithelial cells. Controls without exposure to biofilm after 24 h (A) and 48 h (B), respectively; after 48 h exposure to whole (10-species) biofilm (C) and biofilm without “red complex” species (D). Epithelial cells appear green due to membrane staining with PKH26 green fluorescent cell linker-kit, the nucleus is blue due to DAPI staining and bacteria appear light blue due to FISH staining using the universal bacterial probe EUB338. Due to additional FISH staining with L-Pgin1006-2 and TrepG1_679, *P. gingivalis* and *T. denticola*, respectively (C) are stained red. Scales = 20 µm.

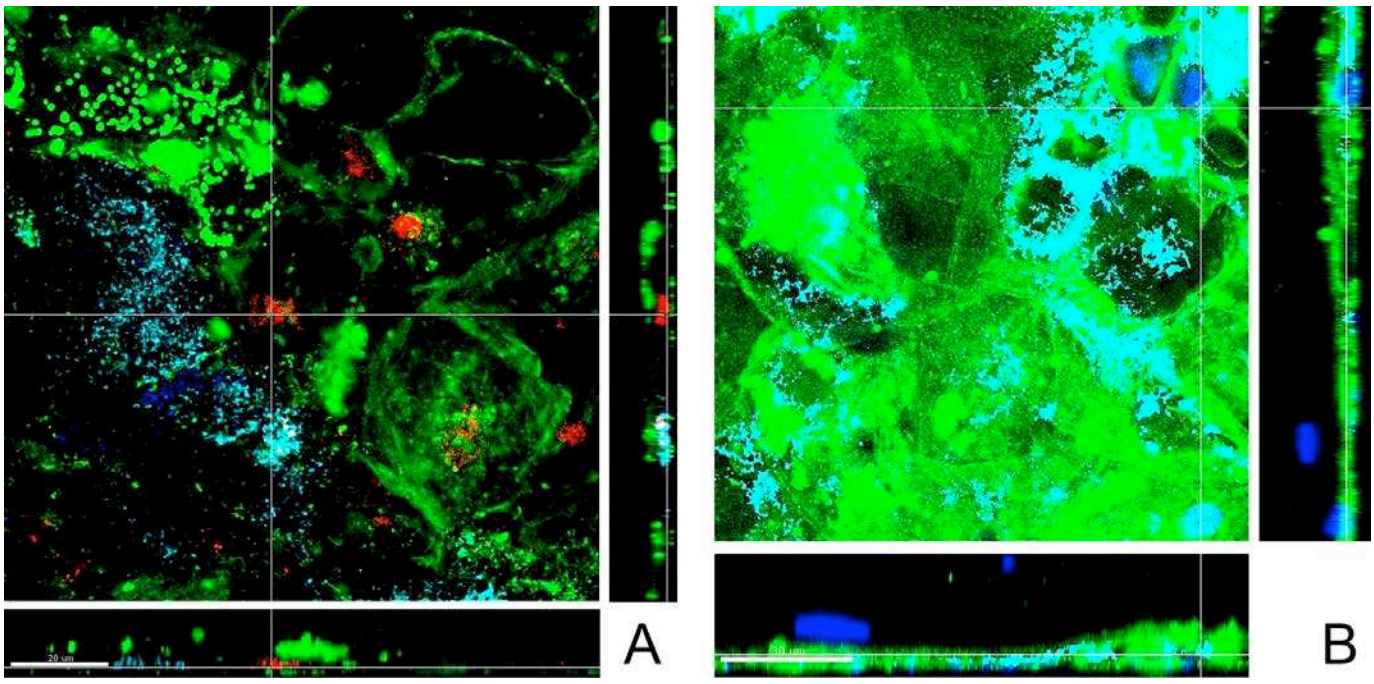

Fig. 5 - CLSM images of gingival epithelial cells after 48-h exposure to the 10-species subgingival biofilm (A) and to the 7-species biofilm without red complex (B). Nuclei appear blue due to DAPI staining, cell membranes green due to staining with PKH26 Green Fluorescent Cell Linker-Kit. Bacteria were stained by FISH using probes EUB338 for total biofilm bacteria (light blue), and Tfor997 for *T. forsythia* (red). Scales = 20 µm (A) and 30 µm (B).

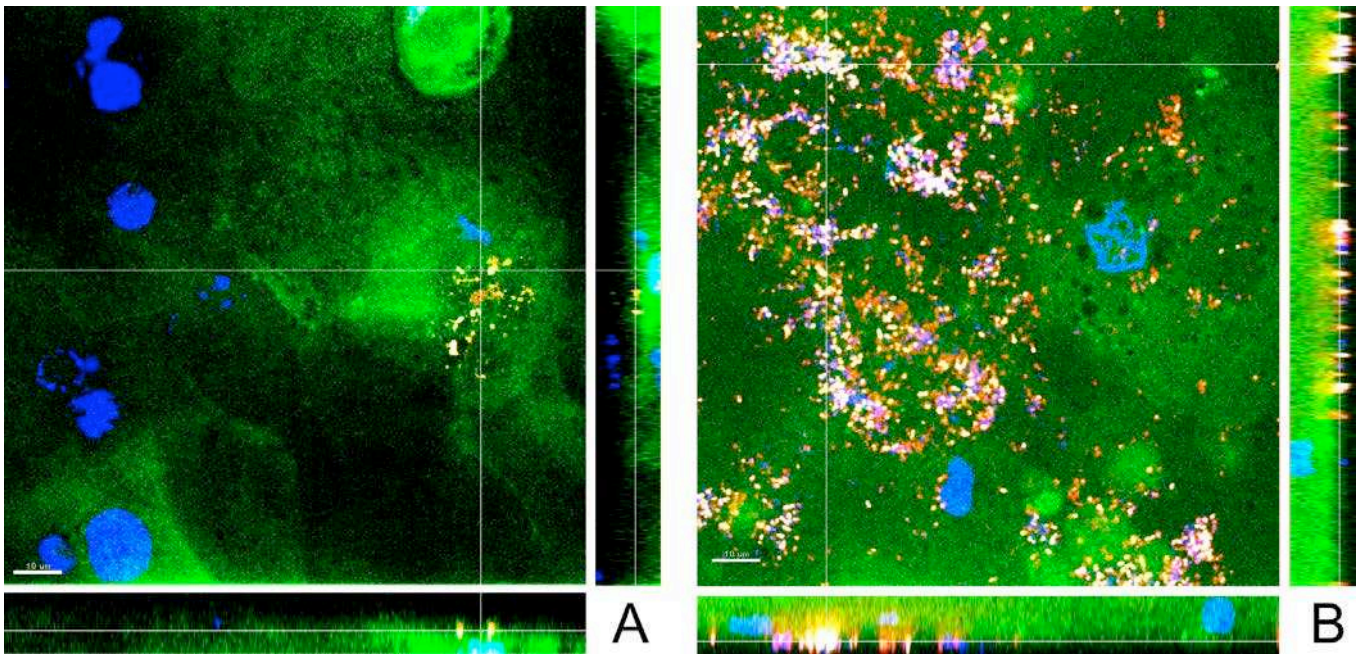

Fig. 6 - CLSM images of gingival epithelial cells after 48-h exposure to the 10-species subgingival biofilm (A) and to the 7-species biofilm without “red complex” (B). Nuclei appear blue due to DAPI staining, cells green due to F-actin staining with phalloidin. Bacteria were stained by FISH using probes EUB 338 for total biofilm bacteria (white), STR405 for total streptococci (red) and Fnuc133c for *F. nucleatum* (red). Scales = 10 µm.